Occurrence and reduction of acytokinesis in leaf protoplast cultures of potato and tobacco. Implications for chromosome number variation.

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CHAPTER 7

SUMMARY AND FINAL CONCLUSIONS.

The studies described in this thesis were carried out to investigate and find means to lower early chromosome number variation during leaf protoplast culture of *Solanum tuberosum* (potato). In a number of plant species, maldistribution of chromosomes had been related to the early occurrence of polynucleate cells. In potato, however, neither the causes of polynucleation nor its impact on poly- and aneuploidization had been investigated. Some general and relevant aspects concerning the encountered variation are introduced in chapter 1.

Chapter 2 describes the cytology of a tetraploid cultivar and a dihaploid clone of potato during protoplast culture. Examination during the first five days revealed relatively low frequencies of normal division and high-frequency development of polynucleate cells. Due to the early onset of cell wall formation, only few of these polynucleate cells could have resulted from spontaneous protoplast fusion. Consequently, the majority had been generated by acytokinesis, i.e. nuclear division without cell division. The nuclear divisions within a polynucleate cell were highly synchronous, giving rise to spindle interaction which resulted in nuclear poly- and aneuploidization. This was supported by the observation that, in general, mitotic cells with 2^N chromosome groups showed equal intracellular ploidy levels, and mitotic cells with a different number (not 2^N) of chromosome groups showed unequal ploidy levels. Aneuploid chromosome groups were found at equal proportions in both categories of mitotic cells. The ability to form cross walls had well been restored after 1-2 weeks of culture, and even multiple cross walls were likely to be formed in the multinucleate cells. This provided evidence for acytokinesis and subsequent spindle interaction to be one of the major sources of chromosome number variation in the protoplasts-derived calli. The influence of this variation on cell proliferation and plant regeneration was argued to be related to the chromosome number of the starting material. Whereas the gain or loss of one or a few chromosomes will often be lethal in species with a small chromosome number, higher chromosome numbers may be capable of buffering such small changes, epecially in a polyploid condition. This would allow some minor forms of aneuploidy to persist during culture and regeneration.

Polyploidization was also found in uninucleate protoplasts/cells, especially in the dihaploid. The overall nuclear polyploidization was also higher in the dihaploid. The difference in nuclear polyploidization between the tetraploid and the dihaploid could have originated entirely from polyploidization at the uninucleate stage. Diplochromosomes, indicating endoreduplication, were only found sporadically.

As the relatively low plating efficiencies (PEs) of both potato genotypes could have been due to inappropriate isolation and/or culture conditions, and because the initially low PEs could entirely be ascribed to the occurrence of acytokinesis, several attempts were made to improve the PE, which might also reduce acytokinesis. Protoplasts of three potato genotypes and one of *Nicotiana tabacum* (tobacco) were isolated and/or cultured under several different conditions. Most of these experiments are described in chapter 3. Although the magnitude of the PEs was genotype-specific, all imposed changes had, if any, a similar effect on the PE for each of the genotypes that were tested. Based on the existing literature, the experiments involved a whole range of potentially beneficial alterations, e.g. the addition of cellobiose, activated charcoal, or bovine serum albumin to the culture medium (KM8p). Nonetheless, substantial improvements in isolation or culture were not achieved.

Cytological studies on two other species are described in chapter 4. The diploid *Solanum bulbocastanum* was taken in order to compare the early polyploidization behaviour of the dihaploid — i.e. haploidized — potato with that of a natural diploid relative belonging to the same genus. A tetraploid tobacco line was taken because it showed high PE, good plant regeneration, and low protoclonal chromosome number variation. Acytokinesis occurred at high frequency in both species, but outer and cross wall formation were better in tobacco. In contrast to all three investigated genotypes of *Solanum*, the mitotic spindles in polynucleate cells of tobacco showed no spatial interaction. Apparently, initial acytokinesis does not affect chromosome number variation in protoplast-derived calli of tobacco.

The diploid *S. bulbocastanum* showed polyploidization similar to the dihaploid clone of potato. In conclusion, the ploidy level itself rather than previous haploidization may have the most important influence on nuclear polyploidization. Occasionally, dissociating diplochromosomes were found during prophase. If this is a general phenomenon in *Solanum* protoplasts, it would mean that endoreduplication can still be held responsible for part of the nuclear polyploidization.

The cytological data obtained after 1, 2, and 3 weeks of protoplast culture clearly confirmed the participation of polynucleate cells in callus formation of both species.

An important observation in the aforementioned studies concerns the size of protoplast-derived cells: after 1 and 2 weeks, single cells were frequently larger than or equal in size to multicellular clumps, especially in the *Solanum* genotypes. In the following investigations, attention was given to possible relations between cytological development and cell size.

For a number of species, immobilization of leaf protoplasts in alginate was known to improve their PE. In chapter 5 it is shown that, in the second week of culture, this procedure also improved the PE of potato protoplasts, which was accompanied by a reduction of acytokinesis. The potato protoplasts showed an improved cell wall development and a slower increase in volume as compared to protoplasts in free suspension. The immobilization had no directly visible effect on tobacco protoplasts.

Whether immobilized or not, their cell wall development was similar to that of the immobilized potato protoplasts, and their volume increase was smaller than that of the freely suspended potato protoplasts. The volume frequency distributions of the developing cells in both culture types indicated the existence of two categories of protoplasts with different growth potential. It was argued that the presence of a rigid cell wall could impede passive expansion in both categories, thereby facilitating cross wall formation, and that it would stimulate active growth, i.e. enlargement, in one category. Wall formation may have been improved by functioning of the alginate matrix as a diffusion barrier for wall polymers. Linkage of these polymers, a necessity for the development of a rigid cell wall, may be carried out less efficiently by potato than by tobacco protoplasts.

The apparent relation between volume increase and the incidence of acytokinesis motivated the study of developmental cytological effects of initial volume reduction. Chapter 6 shows that, despite somewhat lower rates of nuclear division, the evacuolation of potato and tobacco leaf protoplasts resulted in earlier and more frequent cell division; a substantial reduction of acytokinesis was accomplished for both species. The proportional volume increase of the miniprotoplasts was considerably larger than that of the protoplasts, but was insufficient to level the difference within the culture time of 12 days. Compared to protoplasts, miniprotoplasts possess a smaller surface, which may ensure an improved cell wall formation and the above-mentioned consequences thereof. Thus, evacuolation reduces initial acytokinesis. For species/genotypes that are susceptible to spindle interaction, like the presently investigated *Solanum* genotypes, this implies a reduction in the proportion of poly-, aneu-, and/or mixoploid protoplast-derived calli. Consequently, cell proliferation and plant regeneration may be improved, and overall somaclonal variation may be lowered.

In chapters 5 and 6, a clear relation was shown to exist between acytokinetic divisions and cell expansion/growth due to insufficient cell wall formation. Thus, the occurrence of acytokinesis is probably specific for early stages of (leaf) protoplast culture and is not to be generalized for other types of tissue culture. That acytokinesis does not necessarily lead to poly- and/or aneuploidization was shown in tobacco. Its high growth potential and low protoclonal variation may be related to this. In the investigated *Solanum* genotypes, prevention of acytokinesis will cause a considerable reduction in the formation of poly-, aneu-, and mixoploid calli. Unfortunately, the experiments described in chapters 5 and 6 did not fully abolish acytokinesis. In future experiments, a combination of evacuolation and immobilization, preferably in an expansion-limiting gel, may completely eliminate the occurrence of acytokinesis. Through a reduction of chromosome number variation, this would certainly optimize the growth and regeneration potential of potato protoplasts.